Citrus Greening : Overview of the Most Severe Disease of Citrus

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Citrus is the most important fruit crop in the world that is grown commercially in more than hundred countries. In India it is the third major fruit crop after banana and mango and occupies a pride position in the economy of the country. Citrus crop is often affected by many fungal, bacterial and viral diseases which are responsible for severe loss to the growers. Among these diseases, citrus greening is the most devastating one (Garnier and Bové 1983, da Graça 1991). A peculiar yellow shoot symptom of the disease, noted as Huanglongbing (HLB) was first observed in China in late nineteenth century. Subsequently the disease was reported from different countries under different names like citrus die-back from India, mottle leaf disease from Philippines, yellow branch or greening from South Africa etc (Bové and Saglio 1974). Presently the disease is commonly known as citrus greening or Huanglongbing (HLB). The causative organism is a gram-negative, *Candidatus* Liberibacter spp. which belongs to family *Rhizobiaceae.* The disease is associated with citrus decline and cause great production loss all over the world. Citrus greening infected trees are mostly stunted and infected branches start drying as disease progresses. It was also observed that diseased plants were more fragile compared to healthy plants and they are adversely affected by extreme temperatures and moisture. Citrus greening disease was not given serious attention it deserved for many years until it started invading citrus in Florida, USA. The typical disease symptoms were first seen in São Paulo state, Brazil and in Florida, in the years 2004 and 2005, respectively which are the world's largest citrus growing regions.

Biology of the pathogen

Greening was considered to be a virus borne disease till the year 1967 as it was graft transmissible and at that time only viruses were known to spread by grafting. Subsequently association of mycoplasma like organisms (MLOs) with the disease was reported (Doi *et al* .1967, Laflèche and Bové 1970a). Mycoplasma was the new class of bacteria which lacks a defined cell wall and pleomorphic and could thus enter easily in the sieve tube and reside in the phloem. Additionally, the symptoms of citrus greening disease were similar to other MLO associated diseases which lead to strengthen the belief that the agent was MLO (Laflèche and Bové 1970b). However, electron microscope study revealed that the organism consisted of much thicker (25 nm) membrane than typical mycoplasma (7 to 10 nm) suggested that the greening organism contained a definite cell wall (Saglio *et al*. 1971, Garnier *et al*. 1976). Further, with cytochemistry study of the peptidoglycan layer, the causal greening pathogen was confirmed to be gram-negative in nature (Garnier and Bové 1977, Garnier *et al.* 1984). As the pathogen was not cultured on artificial media, its taxonomical classification was based on the 16S rRNA gene sequence instead of traditional methods such as morphology, growth, enzymatic activity (Table 1) (Jagoueix *et al.* 1994). It is grouped into alpha subdivision of proteobacteria, genus *Candidatus* Liberibacter (*Ca.* Liberibacter) in the family *Rhizobiaceae* (Garnier et al., 2000; Garnier *et al*. 1991). Phylogenetic analysis has shown that 'Ca. Liberibacter' spp. as an early branching member of the family (Figure 1) (Jagoueix *et al.* 1994).

Presently three species of Liberibacter are known to be associated with citrus greening and are '*Ca*. L. *asiaticus*' (Las), '*Ca*. *L. africanus*' (Laf), and '*Ca*. L. *americanus*' (Lam) (Villechanoux *et al.* 1992, 1993, Gao *et al*. 1993, Jagoueix *et al.* 1997). The other three members of the genus Liberibacter economically important are, '*Ca*. L. *europaeus*', '*Ca*. L. *solanacearum*' and '*Ca*.

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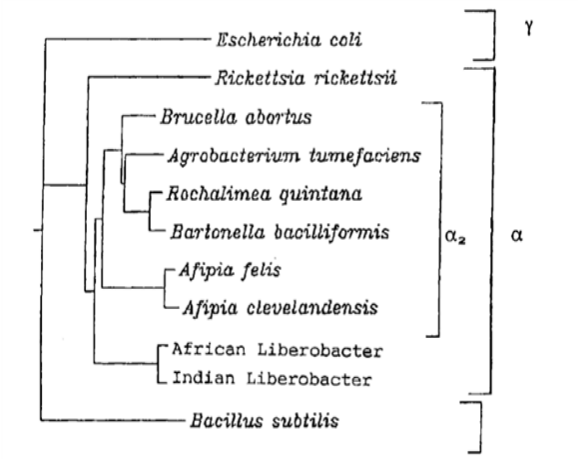

Figure 1. Phylogenetic tree showing relationship between African and Indian Liberibacter, and representatives of α subdivision of the *proteobacteria. Bacillus subtilis* was used as an outgroup (Source: Jagoueix et al., 1994).

L. *crescens*'. Among them '*Ca*. L. *solanacearum*' is responsible for causing serious disease of potato (zebra chip, psyllid yellows), tomato (psyllid yellows) (Abad *et al*. 2009, Nelson *et al*. 2011). '*Ca*. L. *europaeus*' is the new member, found recently in psyllid *Cacopsylla pyri,* an insect vector of pear decline phytoplasma but its pathogenic association with the disease is not clear yet. '*Ca*. L. *crescens'* isolated from mountain papaya plant infected with bunchy top disease has been cultured on artificial media (Leonard *et al.* 2013, Lin 2011). All strains of '*Ca.* Liberibacter' spp. are vectored by insect psylla and phloem-limited, except '*Ca*. L. *crescens*' present in the periphery of phloem and its association with insect vector is not yet confirmed (Raddadi *et al*. 2011). All members of the genus are believed to have been evolved from a common ancestor and in the course of evolution got adapted in different plant hosts (Moran *et al*. 2008, Wernegreen *et al*. 2002). Inside the plant host '*Ca*. Liberibacter' spp. resides and multiplies in sieve tubes of phloem tissue where they come in contact with many sap sucking insects (Moya *et al.* 2008) and could colonize the vector as an alternative host (Leonard *et al*. 2012).

Disease symptoms

Diagnosing citrus greening on the basis of symptoms is quite difficult as none of them are specific, and citrus trees are often affected by additional problems. Moreover, symptoms on the infected tree depend on its age as well as time and stage of infection (Chino *et al*. 1991, Johnson *et al*. 2012). The 'yellow shoots' is considered as typical symptom of the disease in which leaves are partly yellow, partly green, with several shades of yellow, pale green and dark green, blending into each other with no sharp limits between the various shades of color. This symptom known as "blotchy mottle" was described by Lin (1956) in China and McClean and Schwarz (1970) in South Africa. In early stages of the disease yellowing of leaves along the veins and blotchy-mottling were seen and in later stages chlorotic patterns resemble zinc and iron deficiency symptoms (Garnier *et al*.1996). Eventually defoliation and dieback sets in the plant (Hu *et al*. 2011). In the first year of infection blotch mottle symptoms were seen in large, well developed leaves whereas in next year the same tree produced new

Table 1. Evolutionary distances derived from a comparison of 1,253 bases of the 16S rDNA sequences of various bacterial species in the α subdivision of the *Proteobacteria* and, *E. coli* and *Bacillus subtilis* are used as out group (Source: Jagoueix *et al*. 1994).

Taxon	Subdivision of the *Proteobacteria*	Subgroup	Strain Poona	*Afipia clevelandensis*	*Bartonella bacilliformis*	*Brucella abortus*	*Agrobacterium tumefaciens*	*Afipia felis*	*Rochalimaea quintana*	*Rickettsia rickettsii*	*Escherichia coli*	*Bacillus subtilis*
African BLO (strain Nelspruit)			0.0166	0.1388	0.1198	0.1269	0.1273	0.1389	0.1149	0.1838	0.2489	0.2461
Indian BLO (strain Poona)				0.1374	0.1207	0.1264	0.1248	0.1364	0.1167	0.1834	0.2474	0.2407
Afipia clevelandensis	α	α2			0.1324	0.1038	0.1222	0.0217	0.1246	0.1933	0.2581	0.2472
Bartonella bacilliformis	α	α2				0.0712	0.0682	0.1263	0.023	0.1707	0.2617	0.2403
Brucella abortus	α	α2					0.0636	0.0988	0.0648	0.1746	0.2519	0.2284
Agrobacterium tumefaciens	α	α2						0.1191	0.0691	0.1694	0.2627	0.2328
Afipia felis	α	α2							0.1185	0.1970	0.2494	0.2381
Rochalimaea quintana	α	α2								0.1657	0.2572	0.2318
Rickettsia rickettsii	α										0.3102	0.2605
Escherichia coli	γ											0.2734

mottled leaves, but smaller in size than those from the previous year (Bove 2006). In chemical analysis it was found that the symptomatic leaves have higher potassium and lower calcium, magnesium and zinc. Amino acids concentration was also found lowered except proline (da Graca 1991). Excessive starch accumulation was observed in cytopathic study of phloem which leads to necrosis of the tissues. These changes may have direct or indirect effect on symptom development. Root system of infected plant is often underdeveloped due to root starvation (Oberholzer *et al*. 1965, Brlansky 2000). Disease symptoms can also be seen on fruits, mostly which are underdeveloped, small, lopsided, poorly colored and bitter in taste. American and African strains of greening show symptoms at cool conditions (below 25ºC) and disappear above 27ºC under glasshouse condition. On the other hand, symptoms of Asian strain can be visible even under hot condition i.e. above 27ºC (da Graca 2004).

Transmission

Citrus greening disease was first thought to be associated with mineral deficiency and water logging due to its yellow shoot symptoms (Halbert 1998, Sutton *et al*. 2005). But in China the disease was experimentally transmitted to healthy plant using grafting technique which confirmed that the disease is caused by a pathogen (Lin 1956). African greening was also shown to be graft-transmissible by McClean and Oberholzer in the year 1965. Variation in the extent of of graft transmission of the pathogen was observed as it is unevenly distributed in the tree (Xu *et al*. 1988, Halbert 2005). Therefore the plant part and amount of tissue used for grafting is very crucial. Citrus greening disease is not seed transmissible as infected plants have high number of aborted seeds. The disease mostly spread by grafting and insect vectors. Two species of citrus psyllid, *Diaphorina citri* and *Trioza erytreae* have been observed to transmit the greening pathogen. *D. citri* also known as Asian citrus psyllid is responsible to spread Las and Lam in Asian and American continent (Halbert and Nunez 2004, Pelz-Stelinski *et al.* 2010). The vector is heat resistant and can withstand high temperature but sensitive to high rainfall and humidity. On the other hand, *T. erytreae*, African citrus psylla is responsible to spread Ca. Laf in Africa (Halbert *et al*. 2003). The insect is heat sensitive, adult or any of its form can't stay alive above 32ºC and in order to survive they need cool and moist environment (Inoue *et al*. 2009, Mann et al. 2011). Adult insects as well as 4th or 5th instar nymphs are capable of transmission of the pathogen. Acquisition period is 15-30 min for *D. citri,* and 60 min or more for *T. erytreae* with latent period of 10 and 7 days respectively (da Graca, 1991). Climate and type of leaves also play important role in acquiring the pathogen by the vector, for example in winter feeding on old leaves whereas in spring on young flush was observed ideal for pathogen acquisition by the psyllds and are more attracted to diseased plant due to its yellow green color of the symptomatic leaves which increases the probability of pathogen acquisition and transmission by the insect (van Den Berg 1992, Halbert and Manjunath 2004).

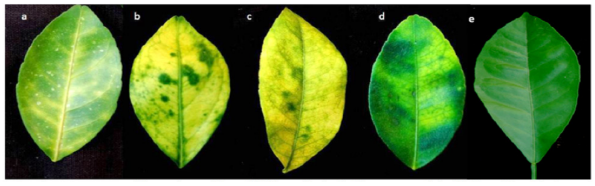

Figure 2. Greening symptoms on different citrus cultivars. (a) Veins yellowing in Cleopatra Mandarin (b) Green islands in Sweet orange (c) Leaf yellowing in Rangpur lime and (d) Yellow mottling in Acid lime, (e) Healthy citrus leaf.

Host range

The host range for greening consist of two types of hosts, one in which the bacterial pathogen multiply and the vector feeds and multiplies, and another alternate host in which the infected psyllids are maintained (Gottwald, 2010; Halbert and Manjunath, 2004). Knowledge of both type plants is very crucial for citrus greening management.

Citrus host

All the species and hybrids of citrus irrespective of their rootstock are susceptible to the greening disease. However symptoms vary from cultivar to cultivar, most

severe found on sweet orange, mandarin, tangelo, and grapefruit whereas less severe symptoms observed on lemon, rough lemon, and sour orange (Bove 2006, Koizumi *et al*. 1992, Liu *et al*. 2011). There is no known resistant citrus species for the disease but some cultivars are more tolerant than others, for example grapefruit is more tolerant than the sweet orange. The pummelo and kumquat (*Fortunella margarita*, Swingle) cultivars were initially considered as resistant, eventually get infected with the disease and start showing mottling symptoms (Bove 2006, Tsai and Liu 2000). This change in resistance is probably due to evolution of pathogen strain over the course of time. The information regarding citrus genotype reactions to the disease is mostly based on symptoms observed in the field conditions at different geographic locations with varying weather conditions which limits the understanding of resistance of different citrus cultivars to citrus greening (Hung *et al.* 2000).

Alternative host

Murraya paniculata (*M. paniculata*) is a preferred host for asian citrus psyllid and considered as an alternative host of greening pathogen. Hung *et al*. (2001) artificially inoculated Las in Chinese box orange (*S. buxifolia*), wood apple (*Limonia acidissima*) and the common jasmine orange (*M. paniculata*). They observed that the pathogen replicates in Chinese box orange and wood apple but not in jasmine orange (Hung *et al*. 2001). In contrast Halbert and Manjunath (2004) found that *M. paniculata* inoculated with Las or Lam showed consistent symptoms like leaf yellowing, defoliation etc. Zhou *et al.* (2007) and many other researchers found symptom generation in jasmine orange.

Non-Rutaceous hosts

There are some other hosts other than *Rutaceae* family which can harbor '*Ca*. Liberibacter' spp. and thus used in greening studies (Zhang *et al.* 2011, Yan *et al*. 2013). It was demonstrated that all three citrus Liberibacters can be transmitted to periwinkle (*Catharanthus roseus*) plants by dodder (*Cuscuta* spp.). It gets colonized and multiplies inside the dodder and infected dodder can be used to transmit the pathogen to citrus, periwinkle plant etc (Garnier and Bove 1983, Hartung *et al.* 2010).

Pathogen virulence

To design effective management strategies understanding of citrus and '*Ca*. Liberibacter' spp. interaction and virulence mechanism is very necessary. Among three Liberibacter species, Las is the most prevalent and destructive. As it is difficult to culture on media its virulence mechanism information is based on genome study.

Flagella

Flagellum is important for phloem-restricted bacteria in order to establish inside the host. However no flagella were observed in any electron micrographs of *Liberibacter*' spp.. A complete set of flagella biosynthesis associated genes were identified in Liberibacter genome (Duan *et al*. 2009, Fagen *et al.* 2014, Lin *et al*. 2011). Interestingly it was found that in planta expression of *fliF, flgI,* and *flgD*, and *motB* genes, involved in motor function is upregulated whereas in the psyllid *flgL, flgK,* and *fliE* genes are over expressed (Yan *et al*. 2013). This expression pattern suggests that Las may have a functional flagellum in some environments. Nevertheless the flagellin protein of Las was recently shown to function as a pathogen associated molecular patterns (PAMP) (Wulff *et al*. 2014).

Lipopolysaccharides

Lipopolysaccharides (LPSs) present on the outer membranes of gram-negative bacteria are made up of lipid A, an oligosaccharide core, and an O-antigen. LPSs contribute to bacterial virulence (Sutcliffe 2010, Narouei *et al*. 2016). Among its components, Lipid A is more conserved than oligosaccharide core and O-antigen. It often triggers plant immune responses, such as oxidative bursts and salicylic acid (SA) accumulation during pathogen-plant interactions (Zipfel and Robatzek 2010). In Las genome two copies of MsbA were detected which is required to synthesize LPS precursors. LPSs acts as PAMP which triggers plant defense system (Hijaz *et al*. 2013).

Phloem aberration

In greening infected plant, necrotic phloem tissue was found due to starch and callose deposition. The starch

deposition in diseased plant was confirmed by aniline blue staining (Kim *et al*. 2009, Schneider 1968). This deposition block the path of photoassimilates due to which typical symptoms like blotchy-mottling of leaves and yellow shoots develop. It was seen that some phloem proteins might also be involved in phloem blockage. When gene expression of diseased citrus and healthy control was compared the PP2 gene was found to be induced (Musetti *et al*. 2010). The gene doesn't express in the early stage of infection as blocking of sieve tubes also stop phloem transportation which leads to nutrient depletion to nearby healthy cells (Hartung *et al*. 2010, Trivedi *et al.* 2012). It is considered as host's defense response to restrict further spread of the pathogen within the sieve tubes. In apple proliferation disease it was found that diseased recovered apple had callose accumulation and phloem-protein deposition in the sieve elements (Koh *et al.* 2011). This might contributed to the recovery by forming physical barriers, preventing the movement of pathogen, '*Ca*. Phytoplasma mali', to healthy parts of plant (Kube *et al*. 2008). In greening infected plants, this callose deposition cause harm not only to the pathogen but also to plant host as it leads to phloem necrosis.

Prophages SC1 and SC2

Complete genome of Las consists of two potential prophage genomes, SC1 and SC2. Both have been suggested to contribute to the pathogenicity of bacterium (Shan *et al*. 2013). Among them SC1 comprises of lytic cycle genes thus after virus particle formation these genes are capable of lytic burst of Las (Ferooz *et al*. 2011, Ibanez *et al.* 2014). Phage particles were observed in the phloem of infected periwinkle using electron microscopy but not found in infected citrus. There might be some factors present in the citrus which inhibit the formation active phage (Fan *et al*. 2012). These prophages might add to the pathogenicity of the bacterium as it encodes multiple virulence factors such as peroxidases which act against reactive oxygen species produced by host system.

Serralysin and hemolysin

Computational analysis of Las genome revealed that gene CLIBASIA_01345 encodes for Serralysin, a putative type I secretion system (T1SS) effector protein. Its expression was upregulated in plant system than in psyllid vector (Sengoda *et al*. 2010). Serralysin is a metalloprotease and first discovered in the culture medium of *Serratia* spp hence the name. It is found to be secreted by many gram negative bacteria to inactivate different antimicrobial proteins and peptides produced by the host (Bland 2007, Castresana *et al*. 2000). Las may also utilize serralysin as its defense to degrade host antimicrobial proteins. These degraded protein used by bacterium as carbon and nitrogen nutrient source for its growth and metabolism. On the other hand hemolysin is produced by animal and insect pathogens to induce cell lysis. It causes leakage of ions, water molecules from the host cell thus leads to cell apoptosis. It was speculated that hemolysin produced by Las may play an important role in its survival in the plant system (Katoh *et al*. 2014, Nakabachi *et al.* 2013).

Pathogen detection

Early and accurate detection of the pathogen is the first and foremost thing to manage the disease. Citrus greening disease is known to affect the citrus crop from over a century. Initially symptom detection in field condition was employed but it is difficult to diagnose the disease primarily based on symptom expression since non HLB and mineral deficiency often produce similar symptom, thus symptom based diagnosis is not practical. Thereafter, various diagnostics tools were developed but it is only from last decade reliable, sensitive and rapid diagnostics are available.

Electron microscope technique

Under field conditions, greening symptoms are difficult to identify, they are mostly distorted or masked by other disease symptoms. Electron microscopy was the first laboratory technique used to identify the pathogen by Lafleche and Bove in year 1970. Cevallos-Cevallos *et al.* (2009) described transmission electron microscopy technique where a thin section of leaf, petiole, stem, bark and root tissue from greening infected plant were used to see the pathogen. The samples first fixed in 3% glutaraldehyde, 0.1 mol L^{-1} potassium phosphate buffer

(pH 7.2) for 4 h at room temperature and then kept overnight in the refrigerator. After that the samples were washed in the same buffer and post-fixed for 4 h at room temperature in 2% osmium tetroxide. Acetone was used to dehydrate the samples and then cut into 90–100 nm sections using diamond knives and then collect on 200-mesh copper grids. These sections then stained with 2% uranyl acetate (aqueous) then post-stained in lead citrate. After that the samples were examine using a Morgani 268 TEM. Electron microscopy was considered as only reliable diagnostic tool before molecular detection techniques were adapted.

Serological detection technique

Monoclonal antibodies (MA) against greening pathogen were developed for enzyme linked immunosorbent assay (ELISA) technique for detection purpose. Thirteen different MA have been produced, first ten MAs were raised using infected periwinkle plant among them two were against the Indian Poona strain, five against a strain from China (Fujian), and three against the South African Nelspruit strain (Garnier *et al*. 1991, Gao *et al*. 1993). These MA are strain specific therefore can not be used for generalized diagnosis of greening (Bove 2006). Serological detection methods are tedious one therefore, molecular based techniques are preferred nowadays.

Molecular detection techniques

Molecular techniques for plant disease diagnosis are more sensitive, reliable and well established as compared to other techniques. Most commonly used methods are polymerase chain reaction (PCR), real-time PCR (q-PCR), flow cytometry, fluorescence in situ hybridization (FISH) and DNA microarrays.

Polymerase chain reaction (PCR)

Greening Bacterium is characterized on the basis of 16S rRNA region, currently three species of the pathogen are recognized Las, Laf, and Lam. A primer set OI1 and OI2c based on 16S rRNA sequence was designed to amplify Las and Laf species whereas another set OA1/OI2c amplifies only Laf isolates (Weisburg *et al*. 1991, Kim and Wang 2009; Li *et al*. 2006). Nucleotide sequence analysis of 16S rRNA region of both the

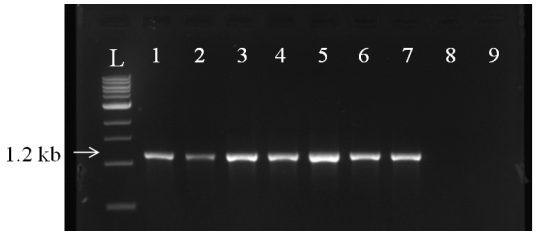

Figure 3. Agarose gel picture of PCR products using primer set OI1/OI2C. L - DNA ladder 1kb, 1 to 7 greening-infected samples, 8 - healthy control, 9 - non template control.

species reveals that Las has only one *Xba*1 restriction site and cut into two fragments of size 520 bp and 640 bp. On the other hand Laf has two *Xba*1 restriction sites and yields three fragments with a size of 520 bp, 506 bp, and 130 bp (Jagoueix *et al*. 1994). There is one more commonly used PCR detection system based on *rpl*KAJL-*rpo*BC operon. Its sequence is slightly different in both the species particularly the intergenic region between genes *rpl*A and *rpl*J is 34 bp larger in the Las than in the Laf (Villechanoux *et al*., 1992; Teixeira *et al*. 2005a). Using forward primer f-*rpl*A2, design in the *rpl*A gene and reverse primer r-*rpl*J5 from the *rpl*J gene, a 703 bp amplicon observed in Asian species and 669 bp in African one. In case of mixed infection, two bands (703 bp and 669 bp) can be seen. A third pathogen species Lam doesn't get amplified with both the PCR system as its nucleotide sequence is different. A new primer set f-GB1/r-GB3 was design based on Lam 16S rRNA region (Teixeira *et al*. 2005, Nageswara-Rao *et al*. 2013a).

Real-time PCR (q-PCR)

qPCR has become the preferred detection method for Ca. Liberibacter spp. (Li *et al*. 2009) as it is more sensitive and rapid than conventional PCR. In qPCR a (fluorescent) reporter is used which binds to the amplified product and report its presence by fluorescence. This generated fluorescence is proportional to amount of product formed (Kim and Wang 2009). Population of pathogen (copy number) can be calculated using qPCR and the technique is so sensitive that it can detect the pathogen even if it is present is low concentration. The lowest reported detection limit for the pathogen is 10

gene copies for 16S rDNA and beta-operon (Teixeira *et al.* 2008). Nageswara-Rao *et al*. (2013a) have developed qPCR using various candidate gene markers specific to Las for early detection of HLB disease.

DNA hybridization

Southern hybridization for the greening pathogen was first developed by Hung and colleagues. A pathogen specific DNA fragment (240 bp) was labeled with biotinylated nucleotides and successfully used for greening diagnosis in various citrus hosts. This DNA probe is specific for Las species and is sensitive enough to detect minute levels of pathogen infection (Hung *et al*. 1999, Nageswara-Rao *et al.* 2013b).

DNA microarray

Microarray technology has been used in various plant, bacterial, fungal and virus diseases to study the gene expression changes after pathogen infection. In this technique transcriptional profiles are compared between infected and healthy plants. First DNA microarray study for 'Ca. Liberibacter' spp. was done by Albrecht and Bowman (Albrecht and Bowman 2008). They compared gene expression of sweet orange plant (*Citrus sinensis* L. Osbeck) in response to pathogen infection using the Affymetrix GeneChipR citrus genome microarray. A total of 33,000 probe sets were used out of them 21,067 were expressed in the leaves and among them 279 and 515 were differentially expressed in 5–9 and 13–17 weeks respectively after inoculation. Interestingly number of mRNA transcripts of phloem-specific lectin PP2-like protein was found to be high. The protein is involved in starch formation and considered as host's defense response to restrict further spread of the pathogen. Using this technique the relationship between pathogen infection and transcriptional changes in host can be examined, which can be further use for disease management (Albrecht and Bowman 2008).

Spectroscopic and imaging technique

PCR is most accurate detection method but it involves identification of infected plants and leaf sampling which is time-consuming. The average accuracy to find disease infected plants in the field from suspected one is 47% to 59% (Futch *et al.* 2009). It was found that by using specific regions in the electromagnetic spectra physiological information of plant can be obtained. This information may be used to differentiate between stressed and healthy plants. Stressed plants can be identified using spectral reflectance of the tree canopy in the visible and infrared regions (Sankaran *et al*. 2010). Visible and near infrared light is being used for disease diagnosis in variety of crops. For example Delalieux *et al.* (2007) detected apple scab disease using hyperspectral reflectance. Naidu *et al*., found out presence of leaf roll associated virus in grapevines using spectrometer in the field condition (2009). Spectrometer was first time used by Sankaran and Ehsani (2011) for citrus greening diagnosis, and subsequently Mishra *et al.* (2011) developed a low-cost, multiband active optical sensor for identification of infected trees. This sensor measured reflectance of tree canopy in 4 bands: 2 visible bands (570 and 670 nm) and 2 near-infrared bands (870 and 970 nm). A new method of high-resolution aerial imaging using unmanned aerial vehicle (UAV) was used by Garcia-Ruiz *et al*. (2013). UAV is capable of taking aerial images at desired resolution by adjusting the flying altitude. The accuracy rate for UAV-based data ranges from 67% to 85% with false negative from 7% to 32%.

Meanwhile, Pourreza *et al.* (2014) developed a less expensive detection method using narrow-band imaging and polarizing filters which target particular symptoms, such as starch accumulation. A special image capturing system was made using narrowband high-power LEDs at 400 and 591 nm, and the reflectance was measured by 2 monochrome cameras. Two simple image descriptors mean and standard deviation of gray values were used and it was seen in image that starch accumulation in the greening infected leaf was highlighted than its visually similar symptoms of zinc deficiency (Pourreza *et al.* 2015).

Volatile organic compounds (VOCs) detection

Plants are known to communicate with other plants and insects using chemical compounds known as VOCs. Upon infection pathogen can change VOCs profile to attract insect in order to spread the disease. Chemical analysis of released VOCs from infected trees can be used

to diagnose the disease (Albrecht and Bowman 2008, Gibney *et al*. 2005). These VOCs can be detected using sensitive analytical methods like gas chromatography - mass spectrometry (GC–MS) and gas chromatography - differential mobility spectrometry (GC–DMS). Aksenov *et al*. (2014) first developed greening detection method based on chemical analysis of released VOCs. A common subset of different chemical compounds differentially expressed in greening infected and healthy plants are given in Table 2 (Aksenov *et al*. 2014).

Loop mediated isothermal amplification (LAMP) based detection

LAMP is novel technique where DNA amplification is done in isothermal condition. In the method amplification was performed with *Bst* polymerase having strand displacement property. The reaction can be carried out in a simple and inexpensive instrument like a water bath at temperatures between 60 to 65°C (Notomi *et al.* 2000; Ghosh *et al*. 2016). LAMP method for greening detection was combined with a Lateral Flow Dipstick (LFD) device for direct visualization of the results (Rigano *et al.* 2014). The assay was highly specific as no cross-reaction was observed and it is able to detect Las from insect as well as plant samples. The technique uses four to six primers that recognize six to eight regions of the *tufB–secE–nusG–rplKAJL–rpoB* gene cluster. For rapid detection LAMP amplification was coupled with a LFD.

	Experimental abundances, au x 10^{-4}		Molar ratios in the mix	
	Uninfected	Infected	Uninfected	Infected
Linalool	8.75	12.5	11.16	11.81
Tridecane	5.28	6.63	6.73	6.28
4-OH-4-Me-2-pentanone	1.21	1.06	1.54	1.00
Hexacosane	8.34	6.53	10.64	6.18
1-Tetradecene	4.03	6.34	5.14	6.01
Tricosane	92.36	64.25	117.83	60.87
Geranial (Citral)	1.79	10.0	2.28	9.48
Tetradecanal	2.52	7.61	3.22	7.21
Phenylacetaldehyde	6.45	8.95	8.23	8.48
Methyl salicylate	5.11	13.2	6.53	12.46
Cumacrene*	0.78	3.69	1.00	3.50
(E)-Beta-ocimene	8.37	5.36	10.68	5.08
Hexadecanol	1.21	1.06	1.54	1.00
Geranyl acetone	25.8	46.1	32.95	43.67

Table 2. Comparison of different chemical compounds in greening infected and healthy plants (Aksenov *et al*., 2014).

Genome Analysis

'Ca. Liberibacter' spp. associated with citrus greening is unculturable therefore acquiring its pure genomic DNA was difficult. Despite this, all three strains are sequenced successfully. Availability of the complete genome helps to study the metabolic and functional capabilities of the pathogen. It provides insights to the biology and pathogenicity of the bacterium.

Metabolic pathway

Genome of Las contains all 14 genes that typically encode NADH dehydrogenase subunits [A-N] which form major component of the respiratory electron transport chain. However, enzymes required for oxidative phosphorylation like polyphosphate kinase (EC 2.7.4.1) along with other related proteins such as cytochrome bc1 complex (EC 1.10.2.2), cytochrome c oxidase, the cbb3-type (EC 1.9.3.1), or the cytochrome bd complex are not present. Interestingly it consist of all four cytochrome O ubiquinol oxidase subunits [I to IV] which is typically expressed by γ-Proteobacteria (the class containing *E. coli* and *Xylella fastidiosa*) whereas α-Proteobacteria (the class containing '*Ca*. L. *asiaticus*') have cytochrome c oxidases as terminal oxidases. Therefore, due to the lack of key enzymes involved in oxidative phosphorylation as well as absence of diverse terminal oxidases, it can be concluded that Las has a limited capacity for aerobic respiration. As the pathogen

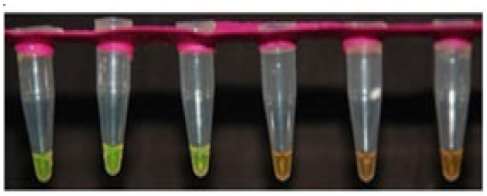

Figure 4. Visualization of LAMP reaction in citrus host using SYBR green I in normal light, tubes 1 to 3 - greening-infected samples, tubes 4 and 5 - healthy control and tube 6 - non template control (Ghosh *et al.*,

has limited aerobic capabilities it generates energy through anaerobic respiration by utilizing nitrogen since several enzymes involved in nitrogen metabolism, such as a NAD+ synthase (EC 6.3.1.5), glutamine synthetase (EC 6.3.1.2), and glutaminase (EC 3.5.1.2), along with those enzymes involved in the glutamate metabolism have been identified (Duan *et al*. 2009).

Las contains enzymes involved the Entner-Doudroff pathway indicating that glycolysis is the major pathway for the catabolism of monosaccharides. Based on the enzymes that are present, Las has the ability to metabolize sugars such as glucose, fructose, and xylulose but not mannose, galactose, rhamnose, or cellulose. Transportation of these sugars in the cell is not clear yet but there is a possibility that the pathogen utilizes ABC transporter proteins for the same. Several obligate intercellular parasites, such as *Chlamydia trachomatis* and *Rickettsia prowazeki,* have developed new energy obtaining system where it scavenge ATP from their host by utilizing unique system, an ATP/ADP translocase (Hatch *et al.* 1982, Winkler 1976). Interestingly, Las encodes for an ATP/ADP translocase along with ATP synthase which facilitates it to synthesize ATP as well as uptake the energy source directly from its host.

Las consist of full set of enzymes necessary for the tricarboxylic acid (TCA) cycle and it may utilize a range of amino acids as energy sources including glutamate, alanine, aspartate, glycine, serine, threonine, methionine, cysteine, arginine, proline, histidine, tyrosine, phenylalanine, and tryptophan. All these amino acids except of proline and tryptophan are found in phloem sap thus it may act as the primary source of these amino acids for the pathogen (Duan *et al.* 2009).

Secretion system

Secretion system is responsible to secret bacterial proteins called effectors into the host cells which are considered as the most important virulence factors of bacterial pathogens. Protein effectors often suppress plant defenses or manipulate developmental processes within the host to benefit the pathogen. Greening bacterium is intracellular and introduced directly into phloem by its insect vector therefore have type I and II secretion system and potentially lack type III and IV systems which play an important role in extracellular pathogen to attack both plant and animal host (Fauvart and Michiels 2008, Felix *et al*. 2008, Munkvold *et al.* 2008). Several complete type I secretion systems were present in Las. It mainly involve two functions, one is defensive, involving multidrug efflux, protecting the bacterium against toxic environmental chemicals, antibiotics produced by other bacteria, and phytoalexins produced by hosts. It is crucial for bacterial survival in host system. The other function is offensive, allowing the secretion of a variety of degradative enzymes and offensive effectors, some of which are antibiotics and others involved in plant or animal pathogenicity. Typically type I secretion systems consist of three protein components, two of which are localized in the inner membrane and one, TolC which traverses both the periplasm and outer membrane (Koronakis *et al.* 2004). The type II secretion system is a membrane bound protein complex responsible for secretion of bacterial toxin and degradative enzymes such as proteases and lipases. All the proteins required for the first step of the type II secretion system, the general secretory pathway (TC 3.A.5.), which is responsible for the export of proteins to the periplasm (Pugsley 1993), were found in the Las genome.

Transport system

Las consist of total 137 transporter proteins among them 9 proteins belong to the channels/pores class of transporters, 24 proteins belong to the electrochemical potential-driven transporters, 92 are primary active transporters, 1 belongs to the group translocators class, and the remaining 11 proteins are members incompletely characterized transport systems class. In between 92 primary active transporters, 40 are ABC transporters, with a wide host range. A typical intracellular bacterium of a similar size consists of on an average of 15 ABC transporters (Davidson *et al*. 2008). There may be possibility that some of these transporters affect virulence, host range, or symptom elicitation, alone or in combination. For example, a phosphate-transport system which is known to mediate the uptake of phosphate has been reported in several studies to be associated with the

virulence of the bacteria (Daigle *et al*. 1995, Mantis and Winans 1993, von Kruger *et al.* 1999). A Zinc transport system (znuABC) acts as virulence factor (Garrido *et al.* 2003). There is a possibility that due to presence of znuABC genes in '*Ca*. L. *asiaticus*', the bacteria absorb Zn ion from the phloem which potentially results in local zinc deficiency, thus Zn deficiency like symptoms develop in the infected plant.

Variation in the Tandem Repeat Number (TRN) between isolates

Earlier, Chen *et al.* (2010) studied the variation analysis of TRN at a genomic locus (CLIBASIA_01645) of '*Ca*. L. asiaticus' strains in Guangdong, China and Florida, USA and observed that Florida bacterial population was dominated by a TRN=5 genotype (84.5%), while the Guangdong bacterial population predominantly contained a TRN=7 genotype, albeit at 47.6%. Katoh *et al.* (2011) identified four tandem repeat loci showing high sensitivity in detection of '*Ca*. L. asiaticus' diversity among isolates from Japan, Taiwan, and Indonesia. In In India, although the disease has been reported from almost all citrus growing regions, knowledge on the molecular variability of the pathogen '*Ca*. L. asiaticus' populations from different geographical regions and cultivars was limited (Ghosh *et al*. 2013). Recently, Ghosh *et al.* (2015) made a detailed study on variability of the Indian '*Ca*. L. asiaticus' based on the tandem repeats at the genomic locus CLIBASIA_01645 and categorized them into four classes based on the tandem repeat copy number (TRN) into; Class I (TRN ≤5), Class II (TRN >5 ≤10), Class III (TRN >10 ≤15) and Class IV (TRN>15). The study revealed that the Indian population of '*Ca*. L. *asiaticus*' is more diverse than reported for Florida and Guangdong populations which showed less diversity. While Florida and Guangdong populations were dominated by a TRN5 and TRN7 genotype respectively, the Indian '*Ca*. L. *asiaticus*' populations with TRN copy numbers 9, 10, 11, 12 and 13 were widely distributed throughout the country. Additionally, TRN2 and TRN17 genotypes were also observed among the Indian '*Ca*. L. *asiaticus*' populations. The predominant '*Ca*. L. *asiaticus*' genotypes from the north-eastern region of India were TRN6 and TRN7 (53.12 %) surprisingly similar to neighboring South China populations. Preliminary results showed absence of preference of citrus cultivars to any specific '*Ca*. L. *asiaticus*' genotype.

Disease management

Citrus crop is cultivated all over the world and nearly all the citrus orchards are affected by greening. Depending on threat level of pathogen and insect vector epidemiology different managementstrategies are getting planned. Quarantine measures were implemented to keep the disease away from non infected places like Australia and the Mediterranean citrus-producing areas. For newly infected citrus-producing areas like California, quarantine and eradication program are being implemented. In these areas early and accurate disease diagnosis is very crucial (Matos *et al.* 2013).

Vector population control

It is well known that wherever there is greening disease there is presence of its vector psyllid insect. Therefore it is important to control the population of insect in order to stop the disease spread. The population can be control led either chemically or using biological agents and both are important for integrated pest management.

Chemical control

It is crucial to spray insecticide 10 to 13 times per year during the flush period. It is important to control insect even on healthy plants and recommended to spray young trees at weekly intervals in rainy season (Tolley 1990, Roistacher 1996). *D. citri* suddenly appears without prior incremental increase in numbers of adults. The population is considered to be high when they reach three nymph and five adults per twig. Recognized chemical pesticides consist of horticultural oils, other natural products and/or organophosphates. In recent studies it was seen that *D. citri* is sensitive to a number of different insecticide classes, including pyrethroids, organophosphates, carbamates, some insect growth regulators (IGRs), horticulture oil etc (Boina *et al*. 2010, Childers and Rogers 2005, Qureshi and Stansly 2009). The level of suppression and residual action of insecticides depends on insect stage and spraying timing. Use of Antifeedants such as neonicotinoids and pymetrozine can reduce the

transmission of Las by inhibiting feeding of plant sap–sucking insects. It is the naturally occurring chemical produced by certain plant to keep insects away (Schwarz *et al*. 1974, Weathersbee and McKenzie 2005). There is report that use of antifeedant reduced the number of phloem salivation events by *D. citri.* Chemical repellents for insects are known to keep away those from plants and these chemicals are found to be effective repellents for *D. citri* in the lab and field condition (Hall *et al*. 2007). However use of antifeedant and repellents are not common practices. They may be used along with pesticides to control the insect populations.

Biological control

A number of fungal pathogens are reported to infect *D. citri,* especially in highly humid conditions for example *Isaria (Paecilomyces) fumosorosea, Lecanicillium lecanii, Beauveria bassiana,* etc. Among them *I. fumosorosea* is considered having most potential impact but there is no published report of its successful use against *D. citri* in the field (Subandiyah *et al*. 2000, Meyer and Hoy 2008, Casique-Valdes *et al.* 2011). There are two well-known parasites of the insect, ectoparasitoid *Tamarixia radiata* (Waterston) and endoparasitoid, *Diaphorencyrtus aligarhensis* (Shaffee *et al*. 1975). Both were first described from the northern Indian subcontinent. It was noted that *T. radiata* apparently is more efficient at parasitizing *D. citri* than *D. aligarhensis* (Tang 1989). Insect predators also used to control *D. citri* population and they are lady beetles, lacewings, syrphids, and spiders. Michaud (2004) reported that the coccinellids *Harmonia axyridis* (Pallas) and *Olla v-nigrum* (Mulsant) were the most abundant predators of *D. citri* followed by the lady bird beetle *Cycloneda sanguinea* (L.) and the anyphaenid spider *Hibana velox* (Becker). Similarly, van Den Berg *et al*. (1992) reported that spiders are important predators of *T. erytreae*, followed by chrysopids, coccinellids, syrphids, hemerobiids, Hemiptera, and predatory mites (van Den Berg *et al*. 1992). However, there is no information about how much they actually reduce psyllid populations.

Plant resistance

Greening resistant commercial citrus cultivars have not been identified till date but some degree of tolerance has been observed in trifoliate orange and some of its hybrids. Trifoliate orange which is graft compatible with *Citrus* spp. and can be used as a rootstock. After graft inoculated with the pathogen, the plant doesn't show any symptoms (Folimonova *et al.* 2009, Alvarado *et al.* 2012). Set of leaf metabolites were compared by Albrecht *et al.* (2016) between Las-infected and healthy green house grown seedlings of the tolerant cultivar US-897 (*Citrus reticulata*×*Poncirus trifoliata*) and susceptible cultivar Cleopatra mandarin (*C. reticulata*), to get the better idea about plant resistance (Albrecht *et al*. 2016). The number of metabolites differentially produced in Las-infected versus uninfected leaves was high in the susceptible cultivar and low in tolerant cultivar US-897. Therefore may be tolerance to the disease did not appear to be associated with the accumulation of higher amounts of protective metabolites in response to Las infection (Albrecht *et al*. 2016). There are some citrus relatives like Indian wood apple (*L. acidissima*) and Murraya species which support Las replication for short span of time but subsequently suppress them completely (Hung *et al*. 2000). The citrus relatives showing resistance/tolerance to the disease can be used in breeding technique to generate greening resistance in citrus cultivars.

Thermotherapy

Thermal therapy treatment is being used to suppress or eliminate disease from plants for decades. A Continuous heat exposure to 40ºC to 42ºC for a minimum of 48 h has been reported sufficient to significantly decrease Las titers or eliminate Las entirely in greening infected citrus seedlings (Kunkel 1936, Hoffman *et al*. 2013). It is also seen that thermal treatment reduces the disease symptoms of citrus trees in groves (Ehsani *et al*. 2013). The main challenge with thermotherapy is that although sufficiently high temperature reduces pathogen load above ground level, temperature could not get increased in roots since soil buffers it. Therefore thermotherapy technique was unable to reduce Las populations in the roots, which then serve as source for re-infection of the canopy (Lopes *et al*. 2013). The effectiveness of thermotherapy on reducing Las populations in roots

needs to be improved for it to become a viable component of integrated HLB management.

Antibiotics

Antibiotics like ampicillin, carbenicillin, cephalexin, oxytetracycline (OTC), penicillin, rifampicin, streptomycin sulfate, and sulfadimethoxine, have been shown to be highly effective to suppress Las population in infected trees (Zhang *et al*. 2014). Recently in Florida use of streptomycin sulfate, OTC hydrochloride, and OTC calcium complex via foliar spray in controlling greening have been approved. Trunk injection of these antibiotics is more efficient but it causes permanent damage to the trunk and lack of suitable device for injection may limits use this technique at commercial scale (Hu and Wang 2016). It was reported that injected plants showed temporary disappearance of symptoms however it is not known that titer of pathogen get reduced or not. It should be studied whether Las will develop resistance against antibiotics or not. This resistance may occur due to horizontal gene transfer (HGT) of mobile genetic elements that carry the resistance genes to degrade the antibiotic among co-occurring bacteria or by mutation of bacterial targets (Chopra and Roberts 2001). But HGT is thought to be less common in intracellular bacteria like Liberibacter, as they are not in contact with external microbial population but are in contact with microbes present in plant sap and insect gut. It remains to be determined whether any of the gut microbes contain genes for resistance to antibiotics. Interestingly Las contains the ABC transporters Msb1 and MsbA2, which have been predicted as multidrug resistance protein. There is a chance that these genes may contribute to the development of resistance to OTC, streptomycin, or other antibiotics. Efforts are being made to improve the application of antibiotics (Li *et al*. 2012, Lin *et al*. 2011).

Use of disease free planting materials

Greening has caused huge amount of citrus tree loss, replanting is crucial for sustaining the citrus production. For replanting, certified greening-free nursery plantlets are required. Horticulturally superior citrus plant should be taken as the mother plant, which is free from greening as well as other diseases, to take budwoods. These budwoods are subsequently employed to generate greening/disease free plantlets, by grafting them on nursery grown rootstock (Gottwald 2010). The nursery, green house should be free from insects as infected psyllid can act as source of inoculation. In addition, instead of replanting one-year-old seedlings, two- or three-year-old seedlings would be more preferable and profitable. These replanted greening free plants will help to manage the disease (Schumann and Singerman 2016, Serikawa *et al*. 2012).

Nutrient enhancement

Foliar applications of micronutrients have been most commonly used in Florida to lessen effects of induced mineral deficiencies. The effectiveness of these treatments has been controversial, and some reports have suggested negative results (Gottwald *et al*. 2012). However Shen *et al*. (2013) reported that enhanced nutrient program has reduced Las population and increased leaf size and weight after at least three years of application. Increase in fruit quality and yield was also observed after foliar nutrient spray. These studies suggest that foliar nutrient spray promotes productivity and over all well being of the plants (Shen *et al*. 2013).

Genetic manipulation in citrus for resistance

Biological breeding between susceptible and resistance/ tolerant species is the most efficient and sustainable approach to produce new varieties that can withstand the disease. However traditional breeding approaches are not much useful as greening affects all most citrus cultivars. Therefore transgenic approaches have been used in citrus resistance against the disease. These approaches includes over expression NPR1 (nonexpressor of pathogenesis related genes 1), antimicrobial proteins (e.g., thionins and plant defensins) or antimicrobial peptides (e.g., cecropin B) (Dutt *et al*. 2015, Stover *et al*. 2013). US Environmental Protection Agency gave permission to a transgenic citrus variety expressing spinach defensins created by Erik Mirkov (Texas A&M University) for field testing of resistance against greening. However consumer acceptance to transgenic varieties is the biggest problem in commercialization of the product. Targeted genome-editing technologies based on zinc

finger nucleases, transcription activator-like effector nucleases, or CRISPR/Cas9–single guideRNA (sgRNA) provide a promising path to improve crops, including generating disease-resistant plants (Nekrasov *et al*. 2013, Belasque *et al*. 2010). Cas9-sgRNA has been used to modify citrus genome to generate disease resistant plants. A disease susceptibility gene CsLOB1 which favors citrus bacterial canker disease, has been modified to generate canker resistant plant (Jia *et al*. 2016a, Jia *et al*. 2016b) and studies in this area of research could be a potential panacea in greening control.

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